Docket No.: 28079/41785
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: David A. Fischhoff et al.	
Application No.: 08/434,105	Confirmation No.: 2627
Filed: May 3, 1995	Art Unit: 1638
For: SYNTHETIC PLANT GENES AND METHOD FOR PREPARATION	Examiner: A. R. Kubelik

DECLARATION UNDER 37 C.F.R. § 1.132

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, JAMES A. BAUM, Ph.D., declare as follows:

I. BACKGROUND, QUALIFICATIONS, AND DISCLOSURE OF INTEREST

I.1 I received my Ph.D. in Genetics from North Carolina State University in 1981. For the following six years, I served as a postdoctoral research fellow at the University of Georgia. I joined Ecogen as a research scientist in 1987. I held several positions within Ecogen, including Principal Research Scientist and Director of *Bacillus thuringiensis* (*B.t.*) research in 1995-1999 and Supervisor of the Ecogen-Monsanto Research & Development Program in 1996-1999. I have been employed as a Research Scientist at Monsanto since 1999. My research has included the discovery of novel insecticidal protein genes from *B.t.*, the engineering of improved insecticidal proteins, the study of DNA-protein interactions, the regulation of gene expression in *Bacillus*, and the optimization of *cry* gene expression in *B.t.* Throughout my career, I have worked alongside and supervised research scientists and postdoctoral fellows engaged in insecticide research. My academic and

professional experience, as well as my research interests, is more fully detailed in my *Curriculum Vitae*, which is attached as Exhibit A.

1.2. Monsanto asked me to comment on the disclosure of Fischhoff et al., U.S. Patent Application No. 08/434,105 ("the Fischhoff application") from the perspective of a research scientist practicing in the field at the time that the Fischhoff application was filed. I was instructed that the Fischhoff application, though most recently filed in 1995, was originally filed in February 1989 (and subsequently re-filed, claiming the benefit of the 1989 filing date). Therefore, my remarks focus on the perspective that an average research scientist would have had in February 1989, unless I state otherwise.

1.3. Monsanto is my current employer and I understand that Monsanto owns the Fischhoff application. Monsanto provided me with a copy of the Fischhoff application; a copy of the Office communication dated February 20, 2008 ("the Action"), in which the U.S. Patent and Trademark Office's patent examiner set forth her current reasons for rejecting the current claims in the Fischhoff application; and a copy of the set of claims of the Fischhoff application that were pending on February 20, 2008, and considered by the patent examiner. (Exhibit B.) I have reviewed these materials for the purposes of this declaration.

II. WRITTEN DESCRIPTION

2.1. I understand that the Patent Office has rejected claims under the "written description" section of the patent laws and taken the position that a person of average skill in the field of the invention would not have recognized that the inventors were in possession of the invention, as presently claimed, at the time the application was filed. In particular, the Action states, "The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention," (Action at p. 3, paragraph 8.)

A. The written description requirement

2.2. It has been explained to me that the goal of the written description requirement is that the patent conveys that an applicant has invented the subject matter which the applicant claims as the invention. I understand that another objective of this requirement is to put the public in possession of the invention that is claimed. It has been explained to me

that an applicant shows possession of the claimed invention by describing in the patent application the claimed invention using descriptive means such as words, structures, figures, diagrams, tables and formulas that fully set forth the claimed invention. I understand that there is no requirement that the language of the claims be found *word-for-word* in the application, as long as the subject matter that is claimed is supported in the application as a whole, through some combination of express, implicit or inherent disclosure.

2.3. I understand that whether the written description requirement is met is viewed from the standpoint of a person of average skill in the field of the invention at the time that the application was filed, with the understanding that the application is written for such persons and the specificity of the disclosure need not be as detailed with respect to features within the knowledge and skill of such persons. Thus, I understand that information which is well known to those in the field of the invention need not be described in detail in the application.

B. Claim terminology directed to "modifying any insecticidal protein coding sequence derived from any *Bacillus* species"

1. Analysis of the Action and rejection

3.1. I understand that one of the Patent Office's concerns is whether the Fischhoff application describes the practice of the invention with respect to any insecticidal protein, or any insecticidal proteins from any *Bacillus* species, or only insecticidal proteins from *Bacillus thuringiensis* (*B.t.*) species, as reflected by the following excerpts from the Action:

> **(a)** Neither the instant specification nor the originally filed claims appear to provide support for modifying any insecticidal protein coding sequence derived from any *Bacillus* species in claims 47, 51, 55, 59, 63, 67, 112, 113, 117 and 119. Neither the instant specification nor the originally filed claims appear to provide support for the concept of the insecticidal protein coding sequence being from any *Bacillus* species. Nowhere in the specification or the originally filed claims is the starting material from any *Bacillus* species, only *B thuringienesis*.

(g) Neither the instant specification nor the originally filed claims appear to provide support for the concept of the starting material being the coding sequence for any insecticidal protein, as in claim 126. The specification indicates that only insecticidal protein coding sequences from *Bacillus thuringiensis* were considered (see pg 16, lines 28, to pg 22, line 24, and originally filed claims 3, 9, 13, and 30).

3.2. The inventors stated in the Fischhoff application, e.g., at pages 16-17, that their invention has very broad applicability to expressing essentially *any* non-plant genes in plant cells. For example, the first paragraph summarizing the "statement of the invention" says, "The present invention provides a method for preparing *synthetic plant genes* which genes express their protein product at levels significantly higher than the wild-type genes which were commonly employed in plant transformation heretofore. In another aspect, the present invention also provides *novel synthetic plant genes which encode non-plant proteins*." (Fischhoff application at p. 16 (emphasis in italics added).) These statements are consistent with the Fischhoff application's broad statements of purpose on page 14 that "it is an object of the present invention to provide a method for preparing synthetic plant genes which express their respective proteins at relatively high levels when compared to wild-type genes" and page 1, first paragraph, that the invention relates to transforming plants to express "a heterologous gene." I interpret a "heterologous gene" to be any gene not originally derived from the host plant species being transformed. The Patent Office seems to agree (at page 6 of the Action) that there is indeed "support for the concept" of this broad scope ("the paragraph spanning pg 16-17 provide support for Bacillus thuringiensis crystal proteins, non-plant proteins, and plant proteins.")

3.3. The Patent Office appears to agree that the Fischhoff application supports the concept of claiming the invention at the narrower level of insecticidal *Bacillus thuringiensis* protein coding sequences. Thus, I understand the question to be whether a reader in 1989 would have understood that the Fischhoff application to provide "support for the concept" of *intermediate scope* method claims – narrower in scope than "all non-plant protein coding sequences" but broader in scope than *Bacillus thuringiensis* insecticidal protein coding sequences, realizing of course that all *Bacillus* protein coding sequences necessarily fall within the scope of all non-plant protein coding sequences.

2. Basis in the Fischhoff application for claims that specify an intermediate genus that is broader than *Bacillus thuringiensis* insecticidal proteins

3.4. In my opinion, it would have been clear to a reader of the Fischhoff application in 1989 that the inventors intended the invention to be more broadly applicable than *B.t.* insecticidal protein coding sequences. As already noted, the Fischhoff application says that the invention is applicable to any non-plant protein coding sequences. Although the Fischhoff application is heavily focused on *B.t.* insecticidal proteins, the Fischhoff application also explicitly says that the inventors' intention in doing so was brevity: "For brevity and clarity of description, the present invention will be primarily described with respect to the preparation of synthetic plant genes which encode the crystal protein toxin of *Bacillus thuringiensis* (*B.t.*)." (Fischhoff application at p. 16.) The Fischhoff application explicitly states, "those skilled in the art will recognize and it should be understood that the present method may be used to prepare synthetic plant genes which encode non-plant proteins other than the crystal protein toxin of *B.t.* …." (Fischhoff application at p. 17.) Thus, a person in the field reading the Fischhoff application would certainly not conclude from the emphasis on *B.t.* insecticidal protein coding sequences that the invention was limited to such protein coding sequences.

a. The Fischhoff application conveys that the invention was considered particularly applicable to any protein coding sequences derived from bacteria of the genus *Bacillus*.

3.5. A researcher who read the Fischhoff application would have also understood that within the category of non-plant protein coding sequences, the inventors considered the invention to be particularly applicable to protein coding sequences from the bacterial genus *Bacillus*. As the Patent Office notes in the Action (pages 4-5), there are many species of *Bacillus* bacteria, so the genus *Bacillus* is broader than *Bacillus thuringiensis*.

3.6. The method claims are directed to removing certain deleterious sequences (e.g., ATTTA sequences and polyadenylation signal sequences) from coding sequences, and the Fischhoff application draws special attention to describing the deleterious sequences in the context of their richness in adenine and thymine nucleotides (see, e.g., Fischhoff application at pages 21-23). A researcher would have appreciated that the specified problem sequences identified in the Fischhoff application and specified in the claims – the ATTTA and the Table II sequences – are themselves (A+T)-rich. In fact, a variation of the

invention focuses on regions of five or more consecutive A or T nucleotides, regardless of the order that they appear. (See, e.g., page 23 and claim 103.) The inventors discovered that coding sequence base composition, particularly the presence of (A+T)-rich regions, limits expression in plants.

3.7. The Fischhoff application points to the *Bacillus* genus as being particularly amenable to genetic modification to enhance protein production in plants, according to the methods of the invention. The application teaches that some *Bacillus* species have genomes among the most rich in adenine and thymine (A+T) bases (see Fischhoff application at, e.g., page 21, lines 1-15 "For example, some *Bacillus* species have among the most A+T rich genomes ....") Because the "problem sequences" associated with low protein production are themselves (A+T)-rich, they are apt to occur with greater frequency in an (A+T)-rich genome.

3.8. The Fischhoff application also discusses the invention with regard to the genus *Bacillus* when characterizing codon usage. "Due to the degeneracy of the genetic code and the limited number of codon choices for any amino acid, most of the "excess" A+T of the structural coding sequences of some *Bacillus* species are found in the third position of the codons. That is, genes of some *Bacillus* species have A or T as the third nucleotide in many codons." (Fischhoff application at p. 21.) The solution taught in the Fischhoff application for eliminating the problem sequences involves substituting codons – and a researcher would understand from the Fischhoff application the advantages of using the degenerate codons for maintaining the same encoded amino acid sequence.

3.9. Thus, reading the Fischhoff application as a whole, a researcher would have understood all of the following:

(i) that the invention was generally applicable to any non-plant protein coding sequence;

(ii) that the problem sequences to target for removal were (A+T)-rich sequences;

(iii) that the genus of bacteria known as *Bacillus* have among the most (A+T)-rich genomes; and

(iv) that the "excess" (A + T) was mostly found in the third position of codons, which were amenable to modification due to the well known degeneracy of the genetic code.

From these and related teachings in the Fischhoff application, a researcher in the field would have understood from the Fischhoff application that protein coding sequences obtained from any species within the genus *Bacillus* were a preferred class of protein coding sequences for improvement according to the invention. The reader would have understood that the inventors intended that the invention apply to all *Bacillus* protein coding sequences as a class, to enhance production in plant cells.

3.10. In the Action the Patent Office asserts that there are at least 192 *Bacillus* species other than *B.t.*, and that "[t]here is no support for any of these species in the specification." (Action at pp. 4-5.) While it is true that there are many species of *Bacillus* and that those species are not listed in the specification, it is also true that there are features common to the genus *Bacillus* that were known to researchers in the field in 1989 and one of those features was highlighted in the Fischhoff application as being relevant to practicing the invention: that some *Bacillus* species have (A + T)-rich genomes, with most of the excess A + T in the third position of codons. A researcher in the field would have understood from the Fischhoff application that the detrimental sequences targeted by the inventors for removal (e.g., ATTTA sequences and polyadenylation signal sequences listed in Table II of the Fischhoff application) could be identified in any *Bacillus* coding sequence, regardless of origin. In other words, the genetic code is universal, and if a researcher were provided with a coding sequence and the teaching of the Fischhoff specification, the researcher would be able to identify the listed detrimental sequences and change them for plant expression, irrespective of the species of origin of the coding sequence. Furthermore, because *Bacillus* genomes were taught to be (A + T)-rich, a researcher would have understood that *Bacillus* genes were more likely to contain (A + T)-rich problem sequences and therefore would be more likely to require modification according the method disclosed in order to achieve expression in plants. The failure to specifically list the other "192 Bacillus species" in the specification does not change my conclusion that the inventors would have recognized the particular suitability of practicing this invention with genes derived from *Bacillus* species other than *B.t.*

b. The Fischhoff application conveys that the invention was considered particularly applicable to pest resistance protein coding sequences and especially applicable to insecticidal protein coding sequences.

3.11. In addition to conveying a preferred (*Bacillus*) and highly preferred (*B.t.*) source for protein coding sequences, the Fischhoff application also conveyed preferred and highly preferred classes of proteins. For example, the Fischhoff application makes specific reference to Example 9 after stating (on page 17) that "it should be understood that the present method may be used to prepare synthetic plant genes which encode non-plant proteins other than the crystal protein toxin of *B.t.*" Example 9 (Fischhoff application pages 96-98) pertains to potato leaf roll virus (PLRV) coat protein, and ordinary researchers would have understood that the purpose of expressing such a protein in plants was pest resistance (see also page 98, last paragraph). Thus, the reader would have understood that the inventors were particularly interested in practicing their invention to improve expression of any coding sequences of proteins that could confer pest resistance when expressed in plants.

3.12. The reader also would have understood from the application that coding sequences for proteins that conferred insect resistance (insecticidal proteins) were highly preferred by the inventors. Example 7 describes, in generic terms, a benefit of introducing two different insecticidal proteins into the same plant. ("In other plants, the production of two distinct insect tolerance proteins would provide protection against a wider array of insects." Fischhoff application at p. 90.) The Fischhoff application elaborates, "Production of two insecticidal proteins in the same plant with different modes of action would minimize the potential for development of insect resistance to *B.t.* proteins in plants." Of course, most of the examples focus on modifying insecticidal protein coding sequences. From passages such as these, a reader would have appreciated that the invention was generally applicable to any heterologous protein contemplated for plant expression, that heterologous proteins that provided pest resistance were a preferred class; and that heterologous proteins that provided insect resistance were a highly preferred class contemplated by the inventors.

c. The Fischhoff application conveys that the invention was considered particularly applicable to insecticidal proteins from *Bacillus*.

3.13. As I explain in the preceding subsections, the Fischhoff application would have conveyed to a reader in 1989 that the invention was broadly applicable to any heterologous protein coding sequences contemplated for expression in plants, and was particularly applicable to protein coding sequences from specific *sources* (e.g., *Bacillus* in general and *B.t.* in particular) and protein coding sequences having specific agriculturally beneficial *properties* (e.g., pest resistance in general or insect resistance in particular). These designations as to preferred *source* and preferred *properties* were not described as mutually exclusive, and to the contrary, most of the examples related to insecticidal proteins from a particular *Bacillus* species (*B.t.*). Accordingly, from reading the Fischhoff application as a whole, a reader skilled in the field would have understood that the inventors contemplated practicing the invention in a number of different ways, including (but not necessarily limited to) the following:

(i) any plant or non-plant protein coding sequence

(ii) any non-plant protein coding sequence

(iii) any *Bacillus* protein coding sequence

(iv) any *Bacillus thuringiensis* protein coding sequence

(v) any pesticidal or insecticidal protein coding sequence

(vi) any insecticidal protein coding sequence from *Bacillus*

(vii) any insecticidal protein coding sequence from *Bacillus thuringiensis*.

Even though the application does not contain an explicit list like this, the application conveys that the inventors contemplated these variations when the application is read as a whole.

3.14. With regard to category (vi) above, it is worth mentioning that species other that *B.t.* were known to have insecticidal properties by 1989, when the Fischhoff application was filed. For example, it was known as early as 1971 that *B. cereus* was toxic to insects. See, e.g., Lysenko, *Folia Microbiol.*, *17*, 228-231 (1972) (Exhibit C), which detailed the pathogenicity of *B. cereus* phospholipase C in moths. Several *Bacillus* species, including *B. popillae* and *B. lentimorbus*, had been identified as pathogenic to *Scarabaeidae* (e.g., the Japanese beetle (*Popillia japonica*)) (see Steinkraus & Tashiro, *App. Microbiol.*, *15*(2), 325-333 (1967) (Exhibit D)). Likewise, the scientific literature identified insecticidal properties

of *B. sphaericus* and *B. laterosporus* against, e.g., mosquitoes and black fly (Aronson et al., *Microbiol. Rev.*, *50*(1), 1-24 (1986) (Exhibit E); Favret and Yousten, *J. Invert. Path.*, *45*, 195-203 (1985) (Exhibit F)). By 1988, the scientific literature included published sequences of mosquitocidal (insecticidal) toxin genes from *Bacillus sphaericus*. (See Exhibit G, Baumann et al. *J. Bacteriol. 170(5)*, 2045-50 (May 1988); see also Exhibit H, Bowditch et al., *J. Bacteriol. 171(8)*, 4178-88 (August 1989) (observing low G+C content for genome of this organism and prevalence of (A + T/U) in third codon position).)

C. Written description for "substantially devoid of polyadenylation signal sequences or substantially devoid of ATTTA sequences"

4.1 The Patent Office alleged, "Neither the instant specification nor the originally filed claims appear to provide support for the concept of producing a coding sequence that is substantially devoid of polyadenylation sequences but not substantially devoid of ATTTA sequences and vice versa, as in claims 47, 51, 55, 59, 63, 67, 112, 113, 117, 119, 120, 122, 124, 126, and 128." (Action at paragraph 8(b), page 6.)

1. The teachings in the Fischhoff application regarding removal of some or substantially all of one or both types of problem sequences.

4.2. Some of the Fischhoff application's direction and guidance regarding removal of ATTTA and/or polyadenylation sequences is found in a paragraph that spans pages 22-23. There, the inventors teach, "In its most rigorous application, the method of the present invention involves the modification of an existing structural coding sequence ('structural gene') which codes for a particular protein by removal of ATTTA sequences and putative polyadenylation signals by site directed mutagenesis of the DNA comprising the structural gene. *It is most preferred that substantially all the polyadenylation signals and ATTTA sequences are removed although enhanced expression levels are observed with only partial removal of either of the above identified sequences*." (Emphasis in underlining and italics added.)

4.3. This short excerpt conveys a lot of information to the reader. First, the underlined, non-italicized portion conveys that the inventors considered removal of both ATTTA and polyadenylation signal sequences to be a highly effective variation of the invention – the most rigorous application of the invention. The italicized, non-underlined excerpt further explains that it is *most preferred* that substantially all of both types of

sequences be removed. It would be quite clear to a reader that removing substantially all of a sequence would result in a modified sequence that was substantially devoid – or in the extreme case completely devoid – of the sequence in question. The paragraph then goes further to explain, in the excerpt that is underlined and italicized, that the invention can be practiced by removing either type of problem sequence, including removal of only some (not all or substantially all) of one type of problem sequence. In the context of the paragraph, the phrase "*only partial removal*" would have been understood to mean removal of some, but not all, of the *occurrences* of a type of sequence. The phrase "*of either of the above identified sequences*" would have been understood in the context of the paragraph to mean *either* the ATTTA sequences, *or* the polyadenylation signal sequences, without the necessity of removing both types of problem sequences. Thus, the underlined and italicized sentence quoted above conveys that all of the following techniques would be effective for enhancing expression, and were all variations of the invention:

(i) removal of some of the ATTTA's, without removing polyadenylation signal sequences;

(ii) removal of some of the polyadenylation signal sequences, without removing any of the ATTTA's.

And referring again to the italicized text, a still more rigorous, and more preferred variation contemplated in the Fischhoff application for practicing the invention was

(iii) removal of substantially all of the ATTTA's, and removal of substantially all of the polyadenylation signal sequences.

4.4. It seems to me that there are many variations in the practice of this invention that are conveyed to the reader, both in the cited text above and in the application as a whole. For example, a reader would understand from the few sentences cited above that the inventors were teaching that the more of either type of problem sequence that was removed, or more specifically the fewer that remained, the better the expression would be. In other words, without spelling it out in word-for-word detail, these sentences convey to the reader other variations of the invention:

(iv) removal of some of the ATTTA's and removal of some of the polyadenylation signal sequences; the reader would have understood that this variation would be better than variation (i) or (ii) because it is closer to the most preferred embodiment - the inventors conveyed that

"removing more" is better (more preferred);

(v) removal of *all or substantially all* of the ATTTA's, without the necessity or removing polyadenylation signal sequences; the reader would have understood that this variation would be better than variation (i) because it is closer to the most preferred embodiment - the inventors conveyed that "removing more" is better (more preferred);

(vi) removal of *all or substantially all* of the polyadenylation signal sequences, without the necessity of removing any of the ATTTA's; the reader would have understood that this variation would be better than variation (ii) because it is closer to the most preferred embodiment -- the inventors conveyed that "removing more" is better;

(vii) removal of *all* of the ATTTA's *and all* of the polyadenylation signal sequences; the reader would have understood that this variation should be better than all of the other variations, because the inventors conveyed that "removing more" of either type of sequence is better, and removing both types of sequence is better than removing only one type of sequence.

I believe that a researcher in the field of the invention would have understood that the inventors contemplated these variations of the invention, as well as perhaps other variations. That is, the cited sentences imply a range of possibilities for practicing the invention, the guiding principles being that removing either type of problem sequence (or both) works and removing more problem sequences is better than removing fewer problem sequences.

4.5. In support of this interpretation, the inventors summarized in the Examples section a large number of experiments where parts or all of insecticidal protein coding sequences were altered by eliminating problem sequences. Sometimes substantially all of one or both types of problem sequence (ATTTA or polyadenylation signal) were removed; sometimes all occurrences of a problem sequence were removed. In at least one variation described in Example 1, using the "BTK240" primer, a construct was made in which only a few occurrences of one type of problem sequence (three polyadenylation signal sequences) were removed. Collectively, the examples convey that the inventors contemplated all of the different variations of the invention enumerated above, and convey that better results can be expected when more occurrences of either (or preferably both) types

of problem sequence are eliminated (when fewer occurrences remain in the modified sequence).

D. Claim terminology relating to the starting sequences encoding portions of two or more insecticidal polypeptides, or two or more polypeptides.

5.1. The Patent Office alleged that neither the specification nor the original claims "appear to provide support for the starting material being sequences encoding portions of any two or more insecticidal polypeptides, as in claims 55 and 67." (Action at paragraph 8(c), pages 6-7.) The Patent Office made related rejections of claims 59, 91-92, and 113-114. Similarly, the Patent Office alleged absence of support "for the starting material being sequences encoding portions of any two or more polypeptides, as in claim 128." (Action at paragraph 8(h), p. 8.)

5.2. In my opinion, it is plain from the Examples that the inventors specifically contemplated practicing the method of the invention on fusions/hybrids of (portions of) coding sequences from two insecticidal proteins, especially fusions of coding sequences of two insecticidal *B.t.* proteins. I note that there are examples of *B.t.* fusions in the Examples section, where the coding sequence was modified according to the invention by removing ATTTA and/or polyadenylation signal sequences. Example 3, for instance, describes a synthetic structural gene encoding a fusion protein derived from *B.t.k.* HD-1 and *B.t.k.* HD-73 (see page 59, lines 13-16, and Figures 3 and 11). A reader in the field would have understood that the inventors intended the Examples and sequences to be representative of categories of subject matter that they intended to claim as their invention.

5.3. Finally, the Fischhoff application conveys to the reader that the method of the invention is suitable for modifying the coding sequence of any non-plant protein. A researcher in the field would understand that the teaching of fusions and hybrids of coding sequences was intended to encompass fusions or hybrids of coding sequences from two plant or non-plant proteins, as well as fusions or hybrids of coding sequences from two insecticidal proteins.

E. Claim language relating to chloroplast transit peptides.

6.1. In paragraph 8(d) of the Action, the Patent Office said, "Neither the instant specification nor the originally filed claims appear to provide support for using coding

sequences for an amino-terminal chloroplast transit peptide or a secretion signal sequence attached to any protein, as in claim 108." I do not agree.

6.2. The Fischhoff application plainly states that aspects of the invention that were described in the context of *B.t.* proteins were described that way for brevity. The reader would not have understood aspects described *in relation* to *B.t.* proteins to be limited to *B.t.* proteins.

6.3. Moreover, the Fischhoff application plainly states that the inventors intended the chloroplast transit peptide and secretory signal sequence modifications to be generally applicable to all protein coding sequences modified according to the invention:

> The DNA construct of the present invention also contains a modified or fully-synthetic structural coding sequence which has been changed to enhance the performance of the gene in plants. In a particular embodiment of the present invention the enhancement method has been applied to design modified and fully synthetic genes encoding the crystal toxin protein of *Bacillus thuringiensis*. The structural genes of the present invention may optionally encode a fusion protein comprising an amino-terminal chloroplast transit peptide or secretory signal sequence (see for instance, Examples 10 and 11).

(Fischhoff application at pages 31-32.)

6.4. The foregoing quote is found *before* the Examples section, in a section titled "Plant Gene Construction." The section by its very title is not about *B.t.* exclusively, but about any structural genes modified according to the invention for plant expression, which can be any non-plant protein. It would be clear to the reader that the inventors only intended modified crystal toxin encoding sequences to be "a particular embodiment" (as stated). The statement about optional inclusion of a chloroplast transit peptide or secretory signal, like the section heading, is general in nature. It expresses optional improvements for *any* modified coding sequence made according to the invention. In fact, the parenthetical use of "for instance" (when referring to Examples 10 and 11) combined with the general title "Plant Gene Construction" further confirms for the reader that the chloroplast transit peptide

or secretory signal sequence is for any modified coding sequence, of which crystal proteins are only one category.

III. DEFINITENESS

7.1. I understand that the Patent Office has questioned whether the meaning of certain claim words or phrases were sufficiently definite that they would be understood by scientists who practice in the field of the invention. I address some of those issues below.

7.2. The definiteness requirement was explained to me as a requirement that the claims define the invention with particularity so persons in the field of the invention would understand what they mean and understand the scope of the invention. I understand that the claim language is interpreted based on the rest of the description of the invention found in the Fischhoff application itself.

A. The phrase "substantially devoid."

8.1. In paragraph 9(a) of the Action, the Patent Office asserts that "Claims 63-68, 112, 117-118, and 126-129 are indefinite in their recitation 'substantially devoid'" because the word "substantially" is a relative term.

8.2. Scientists regularly use relative terms to describe their work, and just because a word is relative does not mean that scientists cannot understand it. Words such as "about" or "approximately" or "substantially" or "most" or "warm" or "cold" abound in the scientific literature. Very often, the context in which the terms are used make them readily understood by scientists who read them.

8.3. In the particular claims in question, the phrase is "substantially devoid," and it is used to describe the number of occurrences of a problem sequence (ATTTA or polyadenylation signal sequences or both) that remain in a modified gene that is made according to the method being claimed. The word "devoid" conveys that something is completely lacking. As I discuss above in some detail, the Fischhoff application explains that the method of the invention works if some of the problem sequences are removed, even if the resulting modified gene is not *completely* free of occurrences of the problem sequence. The term "substantially devoid" would be understood in this context – referring to a situation where one or a few problem sequence remain. The Fischhoff application specifically

indicates that it is preferable to remove substantially all occurrences of a problem sequence. (See pp. 22-23.)

8.4. The Examples in the Fischhoff application provide further guidance for understanding when a modified gene is "substantially devoid" of occurrences of a problem sequence. For instance, Example 2 describes an experiment in which a synthetic insecticidal fragment (amino acids 1-615) of *B.t.k.* HD-1 was made that was devoid of ATTTA sequences (zero) and substantially devoid of Table II polyadenylation signal sequences -- in this instance, only one. Example 3 describes other experiments in which genes encoding chimeric insecticidal proteins were constructed using sequences derived from at least two different *Bacillus* insecticidal protein genes. In one group of experiments, the inventors took the 5' two-thirds of a synthetic HD-1 gene and combined it with the 3' one-third of an HD-73 sequence, modified via site-directed mutagenesis. The resulting construct was devoid of ATTTA, and substantially devoid (reduced in this example from 18 to 2) of occurrences of the polyadenylation signal sequences.

8.5. The Patent Office asks some rhetorical questions about the meaning of substantially devoid, such as, "Does removal of fewer than half of the sequences constitute 'substantially devoid'?" Focusing exclusively on the percentage *removed* is not an appropriate way to evaluate whether a modified sequence is substantially *devoid* because "substantially devoid" means that few are present. If a wildtype gene sequence had only two or one occurrence of a problem sequence, then it would be substantially devoid from the start (without removing any occurrences).[1] By the same token, removal of many occurrences of a problem sequence does not make the resulting sequence "substantially devoid," if many problem sequences are still present. For example, removal of 50 occurrences of ATTTA from a starting sequence that had 100 ATTTA sequences would represent a significant numerical reduction, but no one would conclude that the modified sequence (which still contained 50 ATTTA) was substantially devoid of ATTTA. The claims specify "substantially devoid," not "substantially reduced."

[1] I note that Example 1 says that the HD-1 gene sequence that was modified had 13 ATTTA sequences (see, e.g., Fischhoff application at page 39, last paragraph), not five as alleged by the Patent Office.

B. Reducing occurrences of problem sequences "by substituting sense codons"

9.1. In paragraph 9(b) the Patent Office alleged that the phrase "substituting sense codons" in claims 47, 51, 55, 120, 122, and 124 was indefinite:

> **(b)** Claims 47, 51, 55, 120, 122, and 124 are indefinite in their recitation of "reducing the number of said ATTTA sequences or the number of said polyadenylation signal sequences in the coding sequence by substituting sense codons for codons in the coding sequence". The codons in the coding sequence would already be sense codons, and substituting any sense codon for one in the starting sequence would not necessarily reduce the number of ATTTA or polyadenylation sequences; only substitution with specific sense codons would do so.

(Action at p. 11.)

9.2. I understand the phrase "substituting sense codons" and it would be clear to others in the field of the invention. There are sixty-four codons in the universal genetic code, of which 61 are sense codons – they code for an amino acid. (Table I in the Fischhoff application (pages 26-28) lists the sense codons.) The other three codons code for no amino acid and are sometimes called "stop codons." Because sense codons code for an amino acid, a reader in the field would understand the claim phrase "substituting sense codons in the coding sequence" to mean that the codon that is being introduced (substituted) encodes an amino acid. This clause of the claims excludes substituting a stop codon into the coding sequence, which would have the effect of truncating the encoded amino acid sequence.

9.3. The Patent Office commented that the codons in the coding sequence would already be sense codons. This is correct. Because the method is about "substituting" to reduce occurrence of certain problem sequences, a researcher in the field would understand that the method is specifying substituting a sense codon that is different from the codon in the starting sequence.

9.4. The Patent Office observes that only substitution of specific sense codons would achieve the stated purpose of reducing the number of ATTTA or polyadenylation sequences. This is also correct. However, it does not cast doubt on the meaning of the phrase "substituting sense codons in the coding sequence." The claim simply is specifying two requirements – that the codon that is substituted into the coding sequence be

a sense codon, and that the number of occurrences of the problem sequence be reduced as a result of the substitution. Because a person in the field would be able to determine which of the sense codons eliminate an ATTTA or polyadenylation sequence and which do not, there is no uncertainty in the meaning of "reducing the number of said ATTTA sequences or the number of said polyadenylation signal sequences in the coding sequence by substituting sense codons for codons in the coding sequence...."

C. "Omitting essential steps"

10.1 The Patent Office also alleged that claims 47, 51, 55, 120, 122, and 124 were "incomplete for omitting essential steps, such omission amounting to a gap between the steps." The Patent Office said, "The omitted steps are: making the structural gene comprising a coding sequence that encodes the protein of step (a). As currently written there is no connection between the starting material of part (a) and the making step (c) except the substituted codons -- the rest of the codons of the coding sequence of step (a) are not necessarily involved in step (c)." (Action at p. 11, paragraph 9(c).)

10.2. I agree that the Fischhoff application teaches that, in some variations of the invention, the starting material and the modified gene encode the same protein. (See, for example, original claim 3 on page 115.) However, it is clear to me that the invention does not require this, and the inventors did not *require* this as an essential feature. To provide examples, the Fischhoff application teaches that it may be beneficial to start a protein with "Met-Ala," which may involve mutating the second codon of a protein coding sequence. Original claims 1-2 at page 115 of the Fischhoff application do not require retaining the starting amino acid sequence. By the time that the application was filed, scientists such as myself were able to make mutations in *B.t.* insecticidal proteins that were not activity-destroying, and the invention would be expected to work in such circumstances. The method described in the Fischhoff application focuses on improving expression based on elimination of problem sequences within a coding sequence, and the expression should be improved irrespective of whether the amino sequence is unchanged.

IV. CERTIFICATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that

these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the application or any patent issued thereon.

Date August 14, 2008

James A. Baum, Ph.D.

EXHIBIT A

James Arthur Baum

Research Fellow

Monsanto
700 Chesterfield Parkway West
Chesterfield, MO 63017

Professional History

6/15/87 - 9/1/88	Research Scientist, Ecogen, Inc.
9/1/88 - 9/1/89	Senior Research Scientist, Ecogen Inc.
11/1/89 - 11/1/91	Project Leader: Ecogen/DOW-ELANCO research program
9/1/89 - 3/15/95	Team Leader: Strain Development, Ecogen Inc.
2/1/91 - 2/16/99	Principal Research Scientist, Ecogen Inc.
3/15/95 - 2/16/99	Director of *Bt* Research Principal Research Scientist, Ecogen Inc.
1/24/96 - 2/16/99	Supervisor of the Ecogen-Monsanto research & development program
2/17/99 - present	Research Scientist, Monsanto

Research experience

My research has included the discovery of novel insecticidal protein genes from *Bacillus thuringiensis*, the engineering of improved insecticidal proteins, cloning vector design and construction, the development of an efficient gene transfer system for *B.thuringiensis* employing an indigenous site-specific recombination system, the study of DNA-protein interactions, the regulation of gene expression in Bacillus, and the optimization of *cry* gene expression in *B.thuringiensis*. This work led directly to the development of CRYMAX™ and Lepinox™, two new bioinsecticide products based on genetically-engineered *B.thuringiensis* strains and to patents covering improved strains of *B. thuringiensis*, new insecticidal proteins, and engineered insecticidal proteins. More recently, my research has included alternative insect control strategies employing RNA interference.

Education

1982-1987	Postgraduate	Postdoctoral research fellow with Drs. Norman H. Giles and Mary E. Case, University of Georgia, Athens, GA.

1981	Ph.D.	Genetics North Carolina State University, Raleigh, N.C. Doctoral Dissertation: "Genetic, Biochemical, and Developmental Studies of Maize Superoxide Dismutases."
1976	B.S.	Biology University of Notre Dame South Bend, IN. Honors: Magna cum laude, Phi Beta Kappa

Patents

Shuttle vector for recombinant *Bacillus thuringiensis* strain development. EP 0 533 701 B1. August, 24, 1994.

Bacillus thuringiensis transposon Tn*5401*. U. S. Patent No. 5,441,884. August 15, 1995.

Recombinant *Bacillus thuringiensis* strain construction method. U. S. Patent 5,650,308. July 22, 1997.

Recombinant *Bacillus thuringiensis* strains, insecticidal compositions, and methods of use. U. S. Patent 5,776,449. July 7, 1998.

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Bacillus thuringiensis Tn*5401* proteins. U. S. Patent 5,843,744. December 1, 1998.

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Coleopteran-toxic polypeptide compositions and insect-resistant transgenic plants [MECO164]. WO 00/66742. Provisional filing with U. S. PTO May 4, 1999.

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EXHIBIT B

CLAIMS PENDING AT TIME OF FEBRUARY 2008 PATENT OFFICE COMMUNICATION

1 -46. (Canceled)

47. A method of making a structural gene that encodes an insecticidal protein, the method comprising:

(a) starting with a coding sequence, derived from *Bacillus*, that encodes an insecticidal polypeptide and that contains a plurality of sequences selected from the group consisting of ATTTA sequences and polyadenylation signal sequences listed in Table II;

(b) reducing the number of said ATTTA sequences or the number of said polyadenylation signal sequences in the coding sequence by substituting sense codons for codons in the coding sequence; and

(c) making a structural gene that comprises a coding sequence that includes the codons substituted according to step (b) and is characterized by the reduced number of ATTTA sequences or Table II polyadenylation signal sequences, and that encodes an insecticidal protein.

48. The method of claim 47, wherein step (b) comprises reducing the number of said polyadenylation signal sequences in the coding sequence.

49. The method of claim 47, wherein step (b) comprises reducing the number of said ATTTA sequences in the coding sequence.

50. The method of claim 47, wherein step (b) comprises reducing the number of said ATTTA sequences and the number of said polyadenylation signal sequences in the coding sequence.

51. A method of making a structural gene that encodes an insecticidal protein, the method comprising:

(a) starting with a coding sequence, derived from *Bacillus*, that encodes an insecticidal polypeptide and that contains a plurality of sequences selected from the group consisting of ATTTA sequences and polyadenylation signal sequences listed in Table II;

(b) reducing the number of said ATTTA sequences or the number of said polyadenylation signal sequences in a portion of the coding sequence by substituting sense codons for codons in said portion; and

(c) making a structural gene that comprises said portion with the substitute codons and the reduced number of ATTTA or polyadenylation signal sequences, wherein the structural gene comprises a nucleotide sequence that encodes an insecticidal protein.

52. The method of claim 51, wherein step (b) comprises reducing the number of said ATTTA sequences in said portion.

53. The method of claim 51, wherein step (b) comprises reducing the number of said polyadenylation signal sequences in said portion.

54. The method of claim 51, wherein step (b) comprises reducing the number of said ATTTA sequences and the number of said polyadenylation signal sequences in said portion.

55. A method of making a structural gene that encodes an insecticidal protein, the method comprising:

(a) starting with coding sequences, from one or more structural genes derived from *Bacillus*, that encode an insecticidal polypeptide and that contain a plurality of sequences selected from the group consisting of ATTTA sequences and polyadenylation signal sequences listed in Table II;

(b) reducing the number of said ATTTA sequences or the number of said polyadenylation signal sequences in the coding sequences by substituting sense codons for codons in the coding sequences; and

(c) making a structural gene that comprises the coding sequences with the codons substituted according to step (b) and characterized by the reduced number of ATTTA or polyadenylation signal sequences, wherein the structural gene comprises a nucleotide sequence that encodes an insecticidal protein.

56. The method of claim 55, wherein step (b) comprises reducing the number of said ATTTA sequences in said coding sequences.

57. The method of claim 55, wherein step (b) comprises reducing the number of said polyadenylation signal sequences in said coding sequences.

58. The method of claim 55, wherein step (b) comprises reducing the number of said ATTTA sequences and the number of said polyadenylation signal sequences in said coding sequences.

59. A method of making a structural gene that encodes an insecticidal protein, the method comprising:

(a) starting with an amino acid sequence of an insecticidal protein derived from *Bacillus*, wherein wild-type *Bacillus* gene sequence(s) encoding insecticidal polypeptide(s) from which the insecticidal protein is derived comprise a plurality of nucleotide sequences selected from the group consisting of ATTTA sequence(s) and polyadenylation signal sequences listed in Table II; and

(b) making a structural gene that comprises a coding sequence that:

(i) encodes the amino acid sequence of the insecticidal protein; and

(ii) contains fewer polyadenylation signal sequences listed in Table II or fewer ATTTA sequences, compared to the corresponding coding sequence(s) of the wild-type *Bacillus* gene sequences(s).

60. The method of claim 59, wherein the wild-type *Bacillus* gene sequence(s) comprise a plurality of ATTTA sequences, and wherein the structural gene made according to step (b) contains fewer ATTTA sequences compared to the wild-type *Bacillus* gene sequences(s).

61. The method of claim 59, wherein the wild-type *Bacillus* gene sequence(s) comprise a plurality of said polyadenylation signal sequences, and wherein the structural gene made according to step (b) contains fewer of said polyadenylation signal sequences compared to the wild-type *Bacillus* gene sequence(s).

62. The method of claim 59, wherein the wild-type *Bacillus* gene sequence(s) comprise a plurality of both said ATTTA and said polyadenylation signal sequences, and wherein the structural gene made according to step (b) contains fewer ATTTA sequences and fewer polyadenylation signal sequences compared to the wild-type *Bacillus* gene sequence(s).

63. A method of making a structural gene that encodes an insecticidal protein, the method comprising:

(a) starting with an amino acid sequence of an insecticidal protein derived from *Bacillus*; and

(b) making a structural gene that comprises a coding sequence that encodes the amino acid sequence and that is devoid or substantially devoid of ATTTA sequences, or devoid or substantially devoid of polyadenylation signal sequences listed in Table II.

64. The method of claim 63, wherein step (b) comprises making a structural gene that is devoid or substantially devoid of the ATTTA sequences.

65. The method of claim 63, wherein step (b) comprises making a structural gene that comprises a coding sequence that is devoid or substantially devoid of the polyadenylation signal sequences.

66. The method of claim 63, wherein step (b) comprises making a structural gene that comprises a coding sequence that is devoid or substantially devoid of the ATTTA sequences and devoid or substantially devoid of the polyadenylation signal sequences.

67. A method of making a structural gene that encodes an insecticidal protein, the method comprising:

(a) starting with coding sequences that encode portions of one or more insecticidal polypeptides derived from *Bacillus*; and

(b) combining the coding sequences to form a structural gene that encodes an insecticidal protein,

wherein said coding sequences and the structural gene are devoid or substantially devoid of polyadenylation signal sequences listed in Table II.

68. The method of claim 67, wherein said coding sequences and the structural gene are devoid or substantially devoid of ATTTA sequences.

69. The method according to any one of claims 47, 51, and 55, wherein the structural gene made according to the method is more highly expressed in a dicot plant cell than a structural gene that comprises the starting coding sequence(s) of step (a).

70. The method according to any one of claims 59 and 67, wherein the structural gene made according to the method is more highly expressed in a dicot plant cell than a structural gene that comprises the wild-type *Bacillus* gene sequences encoding polypeptide(s) from which the amino acid sequence of the insecticidal protein is derived.

71. The method according to any one of claims 49, 52, 56, and 60, wherein the structural gene made according to the method contains no more than seven ATTTA sequences.

72. The method according to any one of claims 49, 52, 56, 60, 64, and 68, wherein the structural gene made according to the method contains no greater than one ATTTA sequence.

73. The method according to any one of claims 49, 52, 56, 60, 64, and 68, wherein the structural gene made according to the method contains no ATTTA sequences.

74. The method according to any one of claims 48, 53, 57, and 61, wherein the structural gene made according to the method contains no more than seven of said polyadenylation signal sequences.

75. The method according to any one of claims 48, 53, 57, 61, and 67, wherein the structural gene made according to the method contains no more than two of said polyadenylation signal sequences listed in Table II.

76. The method according to any one of claims 48, 53, 57, 61, 63, and 67, wherein the structural gene made according to the method contains no greater than one of said polyadenylation signal sequences.

77. The method according to any one of claims 48, 53, 57, 61, 63, and 67, wherein the structural gene made according to the method contains no polyadenylation signal sequences listed in Table II.

78. The method according to any one of claims 47, 51, 55, 59, 63, and 67, wherein the structural gene made according to the method contains a (G+C) content of about 50%.

79. The method according to any one of claims 47, 51, 55 and 67, wherein the starting coding sequence(s) of step (a) has (have) an (A + T) content of about 62%.

80. The method according to any one of claims 59 and 63, wherein the wild-type *Bacillus* gene sequences have an (A+ T) content of about 62%.

81. The method according to any one of claims 47, 51, 55, and 67, wherein the starting coding sequence(s) of step (a) are derived from *Bacillus thuringiensis* (*B.t.*).

82. The method according to any one of claims 47, 51, 55 and 67, wherein the starting coding sequence(s) of step (a) are derived from a *Bacillus thuringiensis* (*B.t.*) crystal protein gene.

83. The method of any one of claims 47, 51, 55, and 67, wherein the starting coding sequence(s) of step (a) are derived from a *Bacillus thuringiensis* (*B.t.*) P2 protein or a *B.t. entomocidus* gene.

84. The method according to any one of claims 59 and 62, wherein the wild-type gene sequences are from *Bacillus thuringiensis* (*B.t.*).

85. The method according to any one of claims 59 and 62, wherein the wild-type gene sequences comprise *Bacillus thuringiensis* (*B.t.*) crystal protein gene sequences.

86. The method according to any one of claims 59 and 62, wherein the wild-type gene sequences comprise *Bacillus thuringiensis* (*B.t.*) P2 gene sequences or *B.t. entomocidus* gene sequences.

87. The method according to any one of claims 63 and 66-68, wherein the insecticidal protein is derived from *Bacillus thuringiensis* (*B.t.*).

88. The method according to any one of claims 47, 51, 59, 63, and 67, wherein the insecticidal protein is a *Bacillus thuringiensis* (*B.t.*) crystal protein.

89. The method according to any of claims 47, 51, 55 and 67, wherein the starting coding sequence(s) of step (a) are derived from *B.t. tenebrionus*.

90. The method according to any one of claims 47, 51, 59, and 63, wherein the insecticidal protein is a *B.t.* P2 protein or a *B.t. entomocidus* protein.

91. The method according to any one of claims 47, 51, and 55, wherein the coding sequence(s) of step (a) comprise(s) a hybrid of coding sequences of at least two insecticidal proteins from *B.t.*

92. The method according to any one of claims 59, 63, and 67, wherein the insecticidal protein comprises a hybrid of *B.t.* insecticidal proteins.

93. The method according to any one of claims 47, 51, 55, and 67, wherein the coding sequence(s) of step (a) encode(s) an insecticidal fragment of a *Bacillus* insecticidal protein.

94. The method according to any one of claims 47, 51, 55, and 67, wherein the coding sequence(s) of step (a) encode(s) a full length *Bacillus* insecticidal protein.

95. The method according to any one of claims 59 and 63, wherein the insecticidal protein derived from *Bacillus* comprises an insecticidal fragment of a *Bacillus* insecticidal protein.

96. The method according to any one of claims 59 and 63, wherein the insecticidal protein derived from *Bacillus* comprises a full length *Bacillus* insecticidal protein.

97. The method according to any one of claims 47, 51, and 55, wherein the substituting of sense codons does not change the amino acid sequence encoded by the coding sequence.

98. The method according to any one of claims 47, 51, 55, 59, 63, and 67, wherein the insecticidal protein encoded by the structural gene comprises an amino acid sequence that is identical to the amino acid sequence of an insecticidal protein from *Bacillus*, or an insecticidal fragment thereof.

99. The method according to any one of claims 47, 51, 55, 59, and 67, wherein the insecticidal protein and the insecticidal polypeptide have the same amino acid sequence.

100. The method according to any one of claims 47, 51, 55, 59, and 63, comprising avoiding the introduction of sense codons that are rarely found in plant genomes into the resultant structural gene.

101. The method according to any one of claims 47, 51, 55, 59, and 63, comprising avoiding, in the resultant structural gene, the introduction of sense codons that contain a TA doublet.

102. The method according to any one of claims 47, 51, 55, 59, and 63, comprising avoiding, in the resultant structural gene, the introduction of sense codons that contain a CG doublet.

103. The method according to any one of claims 47 and 55, further comprising reducing the number of regions in the coding sequence(s) with greater than five consecutive adenine and thymine (A+T) nucleotides by substituting sense codons for codons in the coding sequence(s).

104. The method according to claim 51, further comprising reducing the number of regions in said portion with greater than five consecutive adenine and thymine (A+T) nucleotides by substituting sense codons for codons in the portion.

105. The method according to any one of claims 59, 63, and 67, wherein the structural gene comprises a coding sequence that does not contain more than five consecutive adenine and thymine (A+T) nucleotides.

106. The method according to any one of claims 47 and 55, further comprising truncating the coding sequence to yield a truncated structural gene that encodes a truncated protein that retains insecticidal activity.

107. The method according to any one of claims 47, 51, 55, 59, 63, and 67, further comprising attaching a plant promoter to the structural gene.

108. The method according to any one of claims 47, 51, 55, 59, 63, and 67, further comprising including in the structural gene a sequence that encodes an amino-terminal chloroplast transit peptide or a secretory signal sequence.

109. The method according to any one of claims 47, 51, 55, 59, 63, and 67, further comprising attaching to the structural gene a 3' non-translated nucleotide sequence that comprises a plant polyadenylation signal.

110. The method according to any one of claims 47, 51, and 55, wherein the making step comprises performing site directed mutagenesis on a coding sequence from *Bacillus* to make the structural gene.

111. The method according to any one of claims 47, 51, and 55, wherein the making comprises *de novo* synthesis of a fully synthetic structural gene.

112. A method of making a structural gene that encodes an insecticidal protein, the method comprising:

(a) starting with a sequence derived from *Bacillus*, said sequence comprising a coding sequence for an insecticidal protein, or an amino acid sequence of the insecticidal protein; and

(b) making a structural gene that comprises a coding sequence that encodes the amino acid sequence and that is devoid or substantially devoid of ATTTA sequences, or devoid or substantially devoid of polyadenylation signal sequences listed in Table II.

113. A method of making a structural gene that encodes an insecticidal protein, the method comprising:

(a) designing a nucleotide sequence that encodes an insecticidal protein derived from *Bacillus* and that contains a reduced number of ATTTA sequences or Table II polyadenylation signal sequences, compared to wild type *Bacillus* coding sequence(s) from which the insecticidal protein was derived; and

(b) making a structural gene that comprises the nucleotide sequence, that encodes the insecticidal protein, and that is characterized by the reduced number of ATTTA sequences or Table II polyadenylation signal sequences, compared to the wild type *Bacillus* coding sequence(s).

114. The method of claim 113, wherein the insecticidal protein comprises a protein selected from the group consisting of: (a) *Bacillus thuringiensis* (*B.t.*) insecticidal proteins; (b) insecticidal fragments of (a); and insecticidal fusions derived from more than one *B.t.* insecticidal protein.

115. The method of claim 113, wherein the wild-type *Bacillus* coding sequence(s) comprise a plurality of both said ATTTA and said polyadenylation signal sequences, and wherein the structural gene made according to step (b) contains fewer ATTTA sequences and fewer polyadenylation signal sequences compared to the wild-type *Bacillus* coding sequence(s).

116. The method according to claim 113, wherein the structural gene made according to the method is more highly expressed in a dicot plant cell than a structural gene that consists of wild type *Bacillus* coding sequence(s) from which the insecticidal protein was derived.

117. A method of making a structural gene that encodes an insecticidal protein, the method comprising:

(a) designing a nucleotide sequence that encodes an insecticidal protein derived from *Bacillus* and that is devoid or substantially devoid of ATTTA sequences, or devoid or substantially devoid of Table II polyadenylation signal sequences; and

(b) making a structural gene that comprises the nucleotide sequence and that encodes the insecticidal protein, wherein the structural gene is devoid or substantially devoid of ATTTA sequences, or devoid or substantially devoid of Table II polyadenylation signal sequences.

118. The method of claim 117, wherein the structural gene is devoid or substantially devoid of the ATTTA sequences, and devoid or substantially devoid of the polyadenylation signal sequences.

119. A method of making a structural gene that encodes an insecticidal protein, the method comprising:

(a) starting with a coding sequence, derived from *Bacillus*, that contains a plurality of sequences selected from the group consisting of ATTTA sequences and polyadenylation signal sequences listed in Table I, and that encodes an insecticidal protein having an amino acid sequence;

(b) making a structural gene that comprises a coding sequence that:

(i) encodes the amino acid sequence of the insecticidal protein; and

(ii) contains fewer polyadenylation signal sequences listed in Table II or fewer ATTTA sequences, compared to the corresponding coding sequence derived from *Bacillus*.

120. A method of making a structural gene that encodes a protein, the method comprising:

(a) starting with a coding sequence that encodes a polypeptide and that contains a plurality of sequences selected from the group consisting of ATTTA sequences and polyadenylation signal sequences listed in Table II;

(b) reducing the number of said ATTTA sequences or the number of said polyadenylation signal sequences in the coding sequence by substituting sense codons for codons in the coding sequence; and

(c) making a structural gene that comprises a coding sequence that includes the codons substituted according to step (b) and is characterized by the reduced number of ATTTA sequences or Table II polyadenylation signal sequences, and that encodes a protein.

121. The method of claim 120, wherein step (b) comprises reducing the number of said ATTTA sequences and the number of said polyadenylation signal sequences in the coding sequence.

122. A method of making a structural gene that encodes a protein, the method comprising:

(a) starting with a coding sequence that encodes a polypeptide and that contains a plurality of sequences selected from the group consisting of ATTTA sequences and polyadenylation signal sequences listed in Table II;

(b) reducing the number of said ATTTA sequences or the number of said polyadenylation signal sequences in a portion of the coding sequence by substituting sense codons for codons in said portion; and

(c) making a structural gene that comprises said portion with the substitute codons and the reduced number of ATTTA or polyadenylation signal sequences, wherein the structural gene comprises a nucleotide sequence that encodes a protein.

123. The method of claim 122, wherein step (b) comprises reducing the number of said ATTTA sequences and the number of said polyadenylation signal sequences in said portion.

124. A method of making a structural gene that encodes a protein, the method comprising:

(a) starting with coding sequences, from one or more structural genes that encode a polypeptide and that contain a plurality of sequences selected from the group consisting of ATTTA sequences and polyadenylation signal sequences listed in Table II;

(b) reducing the number of said ATTTA sequences or the number of said polyadenylation signal sequences in the coding sequences by substituting sense codons for codons in the coding sequences; and

(c) making a structural gene that comprises the coding sequences with the codons substituted according to step (b) and characterized by the reduced number of ATTTA or polyadenylation signal sequences, wherein the structural gene comprises a nucleotide sequence that encodes a protein.

125. The method of claim 124, wherein step (b) comprises reducing the number of said ATTTA sequences and the number of said polyadenylation signal sequences in said coding sequences.

126. A method of making a structural gene that encodes a protein, the method comprising:

(a) starting with an amino acid sequence of an insecticidal protein; and

(b) making a structural gene that comprises a coding sequence that encodes the amino acid sequence and that is devoid or substantially devoid of ATTTA sequences, or devoid or substantially devoid of polyadenylation signal sequences listed in Table II.

127. The method of claim 126, wherein step (b) comprises making a structural gene that comprises a coding sequence that is devoid or substantially devoid of the ATTTA sequences and devoid or substantially devoid of the polyadenylation signal sequences.

128. A method of making a structural gene that encodes a protein, the method comprising:

(a) starting with coding sequences that encode portions of one or more polypeptides; and

(b) combining the coding sequences to form a structural gene that encodes a protein,

wherein said coding sequences and the structural gene are devoid or substantially devoid of polyadenylation signal sequences listed in Table II.

129. The method of claim 128, wherein said coding sequences and the structural gene are devoid or substantially devoid of ATTTA sequences.

130. The method according to any one of claims 120-129, further comprising attaching a plant promoter to the structural gene.

131. The method according to any one of claims 120-129, further comprising including in the structural gene a sequence that encodes an amino-terminal chloroplast transit peptide or a secretory signal sequence.

132. The method according to any one of claims 120-129, further comprising attaching to the structural gene a 3' non-translated nucleotide sequence that comprises a plant polyadenylation signal.

133. The method according to any one of claims 120-125, wherein the structural gene made according to the method is more highly expressed in a dicot plant cell than a structural gene that comprises the starting coding sequence(s) of step (a).

134. The method according to any one of claims 120-129, wherein the structural gene made according to the method contains no ATTTA sequences.

135. The method according to any one of claims 120-129, wherein the structural gene made according to the method contains no polyadenylation signal sequences listed in Table II.

136. The method according to any one of claims 120-125, wherein the starting coding sequence(s) of step (a) has (have) an (A + T) content of about 62%.

137. The method according to any one of claims 120-127, comprising avoiding the introduction of sense codons that are rarely found in plant genomes into the resultant structural gene.

138. The method according to any one of claims 120-127, comprising avoiding, in the resultant structural gene, the introduction of sense codons that contain a TA doublet.

139. The method according to any one of claims 120-127, comprising avoiding, in the resultant structural gene, the introduction of sense codons that contain a CG doublet.

140. The method according to any one of claims 120-125, further comprising reducing the number of regions in the coding sequence(s) with greater than five consecutive adenine and thymine (A+T) nucleotides by substituting sense codons for codons in the coding sequence(s).

141. The method according to any one of claims 120-129, wherein the structural gene comprises a coding sequence that does not contain more than five consecutive adenine and thymine (A+T) nucleotides.

EXHIBIT C

Folia Microbiol. 17, 228–231 (1972)

Pathogenicity of *Bacillus cereus* for Insects

II. Toxicity of Phospholipase C for *Galleria mellonella*

O. Lysenko

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Received April 22, 1971

A relationship between phospholipase C (EC 3.1.4.3.) (lecithinase C) of *Bacillus cereus* and the pathogenicity of this bacterium for some insect species was mentioned first by Toumanoff (1953). Later the problem was studied in more detail by Heimpel (1955) and by Kushner and Heimpel (1957). It was reported that lecithinase C might play a role in the pathogenicity of *Bacillus cereus* strains and some *Bacillus thuringiensis* strains for sawfly larvae. A more extensive discussion of the

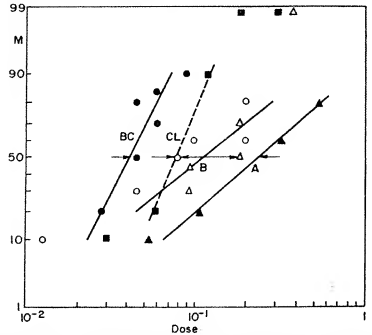

Fig. 1. Determination of LD_{50} of phospholipases C in larvae of *Galleria mellonella*. BC, ●: phospholipase C of *Bacillus cereus* (purified enzyme). CL, ■: phospholipase C preparation of *Clostridium perfringens*. A, ▲: the "crude PLC" of *Bacillus cereus*. B, ○△: PLC active fractions eluted from DEAE-cellulose with 0.5M NaCl (this sample contained all fractions which may be eluted in the range from 0.0 to 0.5M). M, % mortality within one day after injection. Dose, PLC activity in μmol P in 60 min per animal.

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Received April 22, 1971

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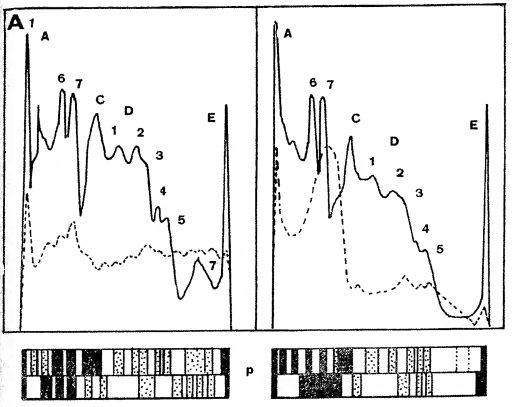

FIG. 2. The changes in the haemolymph of *Galleria mellonella* larvae after injection of *Bacillus cereus* PLC into larval haemocel. A: controls. B: larvae injected with a 10 LD_{50} dose of *Bacillus cereus* PLC. A, C, D, E and 1–7 are numbers of peaks. Solid curve: proteins; broken curve: esterase activity. Disc electrophoresis done according to Davis (1964) with slight modifications: 6.0% acrylamide; 3 mA per tube; gel size 0.5×6.5 cm. Proteins (p) stained with Coomassie brilliant blue R250. Nonspecific esterases (e) were developed by Fast blue RR salts with α-naphthyl acetate as substrate. Gels were scanned in an adapted Zeiss densitometer IR.

problem may be found in Lysenko and Kučera (1971). The toxicities of phospholipase C from *Bacillus cereus* and from *Clostridium perfringens* will be compared here.

Phospholipase C (PLC) produced by *Clostridium perfringens* was a preparation from the Institute of Sera and Vaccines, Prague, under the designation USOL-230. The PLC from *Bacillus cereus* was isolated from *Bacillus cereus* CCEB 665, obtained from Dr. Kandibin, Institute of Agricultural Microbiology, Leningrad, under the label 120a. It was isolated from affected larvae of a noctuid moth. He found it to be highly pathogenic by injection to *Malacosoma neustrium* and *Mamestra brassicae*. The enzyme isolation, characteristics of both preparations and methods for PLC assay were published in a previous paper (Lysenko, 1972). In the present communication the PLC activity is expressed in working units (w.u.), a w.u. being equal to 1 μmol inorganic phosphorus released from lecithovitellin after 60 min at 30°C at pH 7.5.

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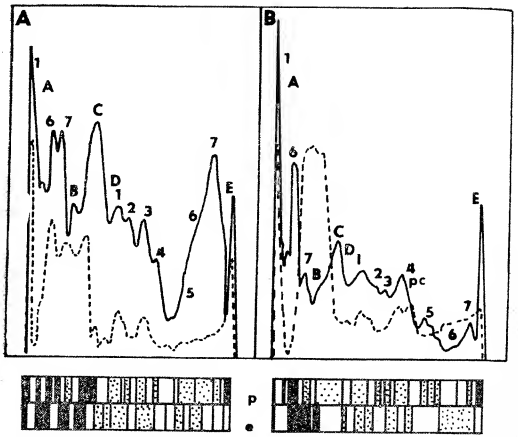
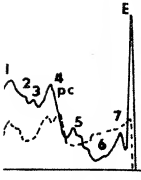
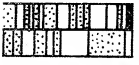


FIG. 3. The changes of haemolymph of *Galleria mellonella* larvae after injections of PLC of *Clostridium perfringens*. Gel concentration 7.5%. The other conditions as well as symbols are the same as used in Fig. 2.

As a model animal, we used the last instar of the wax moth *Galleria mellonella*. The larvae were kept on the semisynthetic diet of Haydak (1936) at 28°C. They were injected with a hypodermic needle (BD,G 27) using an Agla microsyringe. The injected dose ranged from 1 to 10 µl PLC prepared in 0.05M borate or Adam's buffer (Lysenko, 1972). For control, the larvae were injected with the buffer alone. Each dose was applied to 20 larvae and the test was twice repeated. All preparations were filtered through membrane filters and checked for sterility. Mortality was recorded after 18—24 h and the LD_{50} was established by plotting the data on probit paper.

For determining the LD_{50} of phospholipase C from *Bacillus cereus* the following preparations were used: (1) crude PLC which was the concentrated and dialysed precipitate of the culture filtrate, (2) PLC active fractions obtained from this preparation by DEAE-cellulose chromatography on elution with 0.5M NaCl, and (3) the purified PLC preparation as eluted from DEAE-cellulose with NaCl-free buffer (for details see Lysenko, 1972). The toxicities are shown in Fig. 1. From the diagram it may be seen that the LD_{50} of these samples ranged from 4 to 24 . 10^{-2} w.u. The relatively most purified enzyme (BC) displayed a LD_{50} equal to 4 . 10^{-2} w.u., the





njections of PLC of *Clostridium* is are the same as used in Fig. 2.

moth *Galleria mellonella*. lak (1936) at 28°C. They n Agla microsyringe. The d borate or Adam's buffer th the buffer alone. Each ted. All preparations were r. Mortality was recorded he data on probit paper. *cillus cereus* the following ncentrated and dialysed obtained from this prep- th 0.5M NaCl, and (3) the with NaCl-free buffer (for 5. 1. From the diagram it 1 4 to 24 . 10^{-2} w.u. The ual to 4 . 10^{-2} w.u., the

crude PLC (A) 0.24 w.u. and semipurified preparations (B) had a LD_{50} between 8 and 18 . 10^{-2} w.u. It may be concluded that the less purified PLC is less toxic. This may suggest an interference between PLC and some other substance. The phenomenon cannot be simply explained since when inactive fractions were mixed with PLC they neither decreased nor increased the enzyme activity *in vitro* (Lysenko, 1972). Similarly, the slopes of the regression curves (Fig. 1, A, B, compared with BC) show that the response of the animals is not identical.

The LD_{50} of clostridial PLC was 8 . 10^{-2} w.u. per animal. This indicates that it is relatively less toxic than that of *Bacillus cereus*, but bearing in mind that the LD_{50} of *Clostridium perfringens* PLC may vary from strain to strain (as discussed by Oakley, 1970) the difference need not be significant.

The changes found in haemolymph proteins of the larvae upon injecting 10 LD_{50} per animal are shown in Fig. 2 and 3. Electrophoresis was done with the haemolymph of dead larvae (they died within an hour after injection). The changes are seen to be similar with both enzymes, there being a decrease of D_6 and D_7 proteins and a substantial increase of esterase activity located between peaks A_7 and C. PLC activity was not found in the gels. The fact that the changes are somehow associated with phospholipase emerges on comparing the results with those obtained by an action of proteinase (Lysenko and Kučera, 1968). In vertebrates the enzymes were observed to attack mitochondrial phospholipids but it would be mere speculation to extend this finding to insects. Beside the haemolymph protein changes there are alterations in larval haemocytes, as will be discussed in another communication.

The technical assistance of Miss A. Alešová and Mrs. J. Nohavová was highly appreciated.

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EXHIBIT D

APPLIED MICROBIOLOGY, Mar., 1967, p. 325-333

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Milky Disease Bacteria[1]

K. H. STEINKRAUS AND H. TASHIRO

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Received for publication 7 October 1966

A comparative study was made of all available milky-disease species and strains that have been isolated around the world from beetle larvae (family *Scarabaeidae*). Included in the study were *Bacillus popilliae* Dutky, *B. lentimorbus* Dutky, and *B. lentimorbus* var. *maryland* from the United States; *B. euloomarahae* Beard and *B. lentimorbus* var. *australis* Beard from Australia; *B. fribourgensis* Wille from Switzerland; and New Zealand milky disease (Dumbleton). The organisms were classified into three groups: (i) those containing parasporal bodies, including *B. popilliae* Dutky, *B. fribourgensis* Wille, and New Zealand milky disease (Dumbleton); (ii) those without a visible parasporal body and with spore morphology similar to *B. lentimorbus* Dutky, including *B. lentimorbus* var. *australis* Beard; and (iii) those with very tiny spores and no parasporal body, including *B. euloomarahae* Beard and *B. lentimorbus* var. *maryland*. All available milky-disease species and strains were cultivated in vitro on Brain Heart Infusion Agar plates. However, the most fastidious organisms—*B. euloomarahae* and *B. lentimorbus* var. *maryland*—could not be grown until they were passed through a life cycle in larvae of a large scarabaeid beetle infesting rotting wood. Then they remained stable for only one or two subcultures. All the milky-disease organisms produced larger cells in vitro than they did in vivo. The pattern of sugar fermentations was similar for all milky-disease species. It appears that there is a very low percentage of strains of *B. popilliae*, *B. lentimorbus*, and the other milky-disease organisms that have the inherent genetic makeup to permit them to sporulate on artificial media, if conditions are favorable. Among these conditions are a sufficiently high cell population and a reduced oxygen tension. Spores produced in vitro may have a low virulence via the normal ingestion pathway, even though they show apparent virulence when injected directly into the hemocoel.

The milky-disease bacteria consist of a number of species and strains of sporeforming rods which differ morphologically and in their virulence to specific hosts. They have a common characteristic in their ability to complete a life cycle of vegetative multiplication and sporulation within the larval hemolymph of at least one species of *Scarabaeidae*. Infected larvae die, releasing the spores into the soil where they remain viable and ready to infect other larvae. Thus, the milky-disease bacteria are insect pathogens of potential economic importance. They appear to be completely harmless to animals other than their hosts.

The name "milky disease" refers to the clinical condition in which the larval hemolymph becomes so clouded with bacterial cells or spores that it appears milky (Fig. 1).

[1] Approved for publication by the Director of the New York State Agricultural Experiment Station, Geneva, N.Y., as Journal Paper No. 1520.

Milky-disease bacteria have been found all over the world, wherever concentrations of scarabaeid larvae have been studied (Fig. 2). Among these, are *Bacillus popilliae* and *B. lentimorbus*, isolated from Japanese beetle larvae (*Popillia japonica* Newman) in eastern United States by Dutky (7); New Zealand milky disease, found in larvae of *Odontria zealandica* White by Dumbleton (6); *B. euloomarahae*, isolated from larvae of Australian *Heteronychus sanctae-helenae* Blanch, and *B. lentimorbus* var. *australis*, isolated from larvae of Australian *Sericesthis pruinosa* Dalm by Beard (4); *B. fribourgensis*, isolated from *Melolontha melolontha* Linneaus in Switzerland by Wille (28); *B. lentimorbus* var. *maryland*, isolated from Japanese beetle larvae in the United States by scientists at the Moorestown Laboratory of the Entomology Research Division, U.S. Department of Agriculture; and milky-disease organisms found in rhinoceros beetle larvae (*Orycetes* spp.)

in Ceylon and Madagascar (Surany, *unpublished data*).

There are at least four distinct species of spore-forming bacilli and numerous strains that are able to parasitize the larvae of particular species of beetles. They can be divided into three main groups: (i) those apparently related to *B. popilliae* Dutky and containing parasporal bodies; (ii) those similar to *B. lentimorbus* Dutky with no visible parasporal body; and (iii) the milky-disease bacteria with very small spores, similar to *B. euloomarahae* Beard.

Milky-disease organisms exhibit two types of virulence. The first is the ability, when ingested by susceptible hosts, to penetrate, by a mechanism so far unknown, from the gut into the hemolymph where the life cycle is completed. This is the normal pathway of infection. The second type of virulence is rather artificial but has been widely used in milky-disease studies; it is the virulence shown when the organisms are injected directly into the hemocoel, where they then multiply and sporulate. Although strains showing virulence via ingestion may be expected to be equally invasive when injected, strains virulent via injection are not necessarily virulent via ingestion (27).

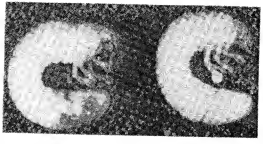

FIG. 1. *Healthy (left) and milky-diseased (right) European chafer larvae. From Tashiro and Steinkraus (27).*

The life cycle of *B. popilliae* Dutky is shown in Fig. 3. The other milky disease species and strains have similar life cycles.

In a search for an organism with the greatest virulence that would be a promising pathogen for biological control of the European chafer (*Amphimallon majalis* Razoumowsky), all available species and strains of milky disease organisms were collected and their virulence tested (27). By injection, several strains of *B. popilliae* and the Amphimallon and Maryland variety of *B. lentimorbus* and *B. euloomarahae* produced infection in over 70% of the larvae. Via ingestion, neither Maryland variety nor *B. euloomarahae* produced infection.

FIG. 2. *Countries in which milky diseases have been found.*

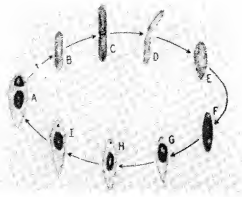

FIG. 3. *Life cycle of Bacillus popilliae Dutky. Adapted from Dutky* (7).

The various species of milky disease bacteria and a number of strains were compared morphologically, biochemically, and biologically in an attempt to compare their life cycles in vivo and in vitro.

MATERIALS AND METHODS

The following cultures of milky disease microorganisms were used in the study: *B. popilliae* Dutky, strain regular type A; *B. popilliae* Dutky, strain DeByne; *B. popilliae* Dutky, strain Majalis; *B. lentimorbus* Dutky, strain Amphimallon; *B. lentimorbus* var. Maryland; *B. lentimorbus* var. *australis* (Beard); *B. fribourgensis* Wille; *B. euloomarahae* Beard; and New Zealand milky disease (Dumbleton).

The spores previously removed from diseased larvae were washed from glass slides on which they had been smeared and dried, suspended in sterile distilled water, and standardized to known concentrations of spores per unit volume with a hemocytometer. Suspensions (a volume of 0.003 ml/grub) were injected into the hemocoel of third-instar European chafer grubs with a tuberculin syringe mounted in a Dutky-Fest microinjector (Dutky and Fest, U.S. Patent 2,270,804). Each larva received roughly 100,000 spores.

Aluminum cake pans (19 by 28.5 cm, 3.8 cm deep) fitted with covers were used to hold the grubs. Each pan was filled to near capacity with moist screened soil containing seed of white sweet clover and domestic rye grass that would serve as food when it germinated. A metal cross-section grid inserted in each pan provided either 40 or 50 compartments, each enclosing 7.6 or 5 cm^3 of soil, respectively. Immediately after inoculation, one grub was placed in each compartment, to prevent cannibalism. The pans were held at 26.7 C. Larvae were sacrificed daily and examined microscopically to determine the course of infection. Grubs were surface-sterilized in 0.05% sodium hypochlorite solution and aseptically bled into 5 ml of sterile water. The cell suspensions were cultured to compare growth of the various species and strains in vivo and in vitro.

Cell suspensions were inoculated on the surface of Brain Heart Infusion Agar (Difco) by use of sterile cotton swabs. The cultures were incubated at 30 C for 4 to 14 days. As soon as vegetative growth was apparent, cells were examined under the phase microscope.

To determine patterns of sugar fermentation, the various species and strains of milky disease organisms were inoculated into a basal medium containing tryptone, 10 g; yeast extract (Difco), 6 g; K_2HPO_4, 1 g; soluble starch, 10 g; and water, 1 liter.

The following sugars, sterilized separately by filtration through sterile Seitz filters, were added individually to the basal medium in a 1% concentration: stachyose, arabinose, xylose, rhamnose, glucose, fructose, galactose, mannose, lactose, maltose, sucrose, and raffinose. After 4 and 7 days at 32 C, the cultures were checked for turbidity and changes in *p*H.

Those milky disease organisms germinating and growing on artificial media were tested for ability to sporulate by the methods of Steinkraus and Tashiro (25), Rhodes, Roth, and Hrubant (16), and Costilow, Sylvester, and Pepper (5).

Photomicrographs were made of the microorganisms as they passed through their life cycles in the host. Cells cultured on artificial media were also photographed and compared.

RESULTS AND DISCUSSION

In vivo life cycles. The life cycles of the various milky disease species and strains in vivo are shown in Fig. 4 as observed, unstained, under the phase microscope in wet mounts. It is seen that all the milky disease organisms so far isolated are sporeforming rods.

The New Zealand milky disease bacillus and *B. fribourgensis* both have parasporal bodies and, thereby, show a direct morphological relationship to *B. popilliae* and its various strains. The parasporal bodies vary in size, even among strains of *B. popilliae*. It is not unusual to find parasporal bodies at both ends of the sporangium. *B. fribourgensis* characteristically shows a rather long tail. The New Zealand milky disease bacillus has a round paraspore, whereas paraspores in some strains of *B. popilliae* are triangular. The remaining species of milky disease organisms do not contain a visible parasporal body and would appear to be related to *B. lentimorbus*. However, two of the organisms, *B. euloomarahae* and *B. lentimorbus* var. *Maryland*, have very tiny spores (Table 1). The rods also are smaller than those produced by the other milky disease organisms.

Spores produced in vivo are shown in Fig. 5. The longer tail on *B. fribourgensis* is quite apparent. Of the two species with very small spores, *B. euloomarahae* has the longer sporangium. Free spores have not been generally observed. The spores of *B. lentimorbus* var. *australis* seem to

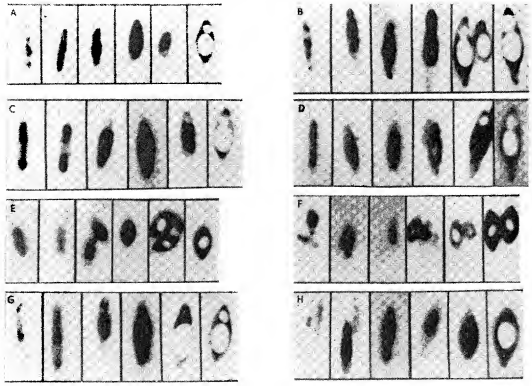

FIG. 4. *Life cycles of milky-disease strains in vivo (European chafer). (A) Bacillus popilliae, strain regular type A; (B) New Zealand (Odontria); (C) B. popilliae, strain DeBryne; (D) B. fribourgensis; (E) B. lentimorbus var. Maryland; (F) B. euloomarahae; (G) B. popilliae, strain Majalis; and (H) B. lentimorbus, strain Amphimallon.* × *2,935.*

TABLE 1. *Comparison of the physical measurements of milky-disease bacilli produced in vivo*[a]

Organism	Dimensions unstained (microns)		
	Sporangium	Spore	Paraspore
Bacillus popilliae	1.6 × 5.5	0.9 × 1.8	0.5 × 0.5
B. fribourgensis	1.4 × 7.1	1.3 × 2.2	0.8 × 0.8
New Zealand milky disease	1.5 × 5.0	0.9 × 1.8	0.9 × 0.9
B. lentimorbus	1.0 × 3.0	0.9 × 1.8	None
B. lentimorbus var. *australis*	1.0 × 3.5	0.9 × 1.9	None
B. euloomarahae	0.7 × 3.2	0.2 × 0.4	None
B. lentimorbus var. *Maryland*	0.7 × 2.8	0.1 × 0.3	None

[a] Based on data in the literature and observations in our laboratory.

have the least obvious sporangia of the milky disease organisms.

Vegetative growth. Vegetative cells of a number of species grown in vivo and in vitro are compared in Fig. 6. The vegetative cells produced in vitro generally are longer than those produced in vivo. There is a tendency to development of involution forms (23).

Beard (2) suggested that the bacteria may penetrate into the hemocoel through the Malpighian tubules. We have found that the first cell produced by the germinating spore frequently has an arrowhead at the emerging end (Fig. 7 and 8). This may provide the mechanical structure needed by the microorganism to pass from the gut to the hemocoel.

It has been possible to germinate the spores and grow the vegetative cells of all milky disease organisms in vitro. The microorganisms are of varying degrees of fastidiousness in their growth requirements. All are aerobic sporeforming rods. The two organisms most difficult to cultivate on aritificial media were *B. euloomarahae* and *B. lentimorbus* var. Maryland. Freshly removed

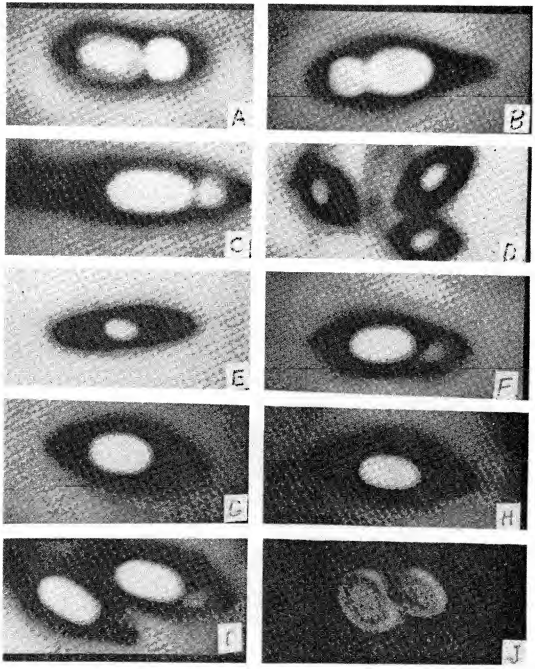

FIG. 5. *Comparison of milky disease spores. (A) New Zealand; (B) Bacillus popilliae, strain regular A; (C) B. fribourgensis; (D) B. lentimorbus var. Maryland; (E) B. euloomarahae; (F) B. popilliae, strain DeBruyne; (G) B. lentimorbus, strain Amphimallon; (H) B. lentimorbus, strain regular B; (I) B. popilliae, strain Majalis; and (J) B. lentimorbus var. australis.* × *15,000.*

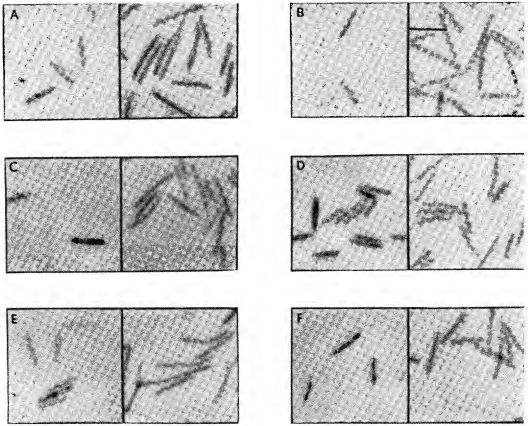

FIG. 6. *Comparison of vegetative cells grown in vivo (left) and in vitro (right). (A) Bacillus popilliae, strain regular A; (B) New Zealand (Odontria); (C) B. popilliae, strain DeBryne; (D) B. fribourgensis; (E) B. popilliae, strain Majalis; and (F) B. lentimorbus, strain Amphimallon.* × *2,935.*

from milky-diseased European chafers (*Amphimallon majalis* Razoumowsky), neither would germinate or grow in vitro. However, by injecting them directly into the hemocoel of large scarabaeid beetle larvae, which are frequently found in rotten wood stumps and which are not normal hosts for these milky disease bacteria, a cycle of in vivo vegetative growth with profuse sporulation was obtained. Spores produced in these larvae were able to germinate and grow for one or two transfers in vitro. Surprisingly, the pattern of sugar fermentation appears to be similar in all milky-disease organisms so far studied. Glucose, fructose, galactose, mannose, and maltose are generally fermented.

On adequately buffered media, where decrease in *p*H is not responsible for loss of viability, vegetative cells generally reach a peak population and then die rather rapidly. This is probably related to the fact that the milky-disease bacteria are deficient in catalase, (23) and peroxidase (12), systems which would enable them to get rid of hydrogen peroxide as it is produced. Within the host, a catalase system is active in the hemolymph and may provide a means for removing hydrogen peroxide as it is produced. Costilow, Sylvester, and Pepper (5) found that hydrogen peroxide is very lethal to the vegetative cells. Addition of catalase or reducing agents, such as ascorbic acid, to the artificial media have not proven to be effective in extending viability or promoting sporulation (5, 23).

Costilow et al. (5) and Rhodes et al. (17) demonstrated that it is possible to achieve population densities of vegetative cells of roughly one-tenth those obtained in the larval hemolymph. We have achieved high cell population densities by growing the organisms on the surface of a solid medium (24, 25). Since the colonies contain only cells, their density in cell pastes is even higher than those achieved in larval hemolymph.

Sporulation. In vitro production of spores or

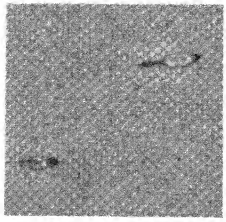

FIG. 7. *Spore germinating, showing the arrowhead-like structure.*

sporelike bodies by *B. popilliae* and *B. lentimorbus* has been reported (5, 9, 15, 16, 24, 25).

Steinkraus and Tashiro (25) and Steinkraus (24) used mainly inocula consisting of spores or vegetative cells (or both) produced in vivo. Sporulation in vitro appeared to be related to obtaining a high concentration of vegetative cells on a growth medium and then further concentrating the cells as a paste on a second medium on which further vegetative growth was impossible. This resulted in a variable but generally low percentage of the vegetative cells sporulating. Only a very few strains had the inherent ability to sporulate rather profusely in vitro by the two-stage procedure.

Rhodes, Roth, and Hrubant (16) advanced the knowledge of sporulation of *B. popilliae* by finding a specific strain (NRRL B-2309S) that could be cultivated in liquid culture and that would form spores, even after numerous subtransfers, under specified conditions. These conditions included transfer of the cells to the surface of a second medium, in which acetate was provided as a carbon source instead of glucose. It was necessary to provide specific concentrations of agar and yeast extract, and it was essential to have fewer than 30 colonies per plate in order to obtain spores.

Hrubant, Rhodes, and Hall reported (Bacteriol. Proc., 1965, p. 7) increased sporulation of the same strain and limited sporulation of three other strains by the Rhodes, Roth, and Hrubant (16) method, when it was modified by (among other changes) autoclaving the ingredients separately and by adding a thin, washed agar overlay to the plates before colony development.

Haynes and Rhodes (9), using the same strain as Rhodes, Roth, and Hrubant (16), found that it would sporulate in the liquid medium in which it was growing vegetatively, if certain types of activated carbon were added to the medium. At least part of the effect of the activated carbon appeared to be a preservation of the viability of the vegetative cells for a longer period, enabling some of them to continue on to the spore stage. The activated carbon may have absorbed growth or sporulation inhibitors. Thus far, apparently only the one strain sporulates by this technique, and data on the yield of spores are fragmentary. However, the yield of spores would appear to be low. Heat resistance of the spores has not been determined. So far, no information has been published on the spores' virulence via ingestion.

Costilow, Sylvester, and Pepper (5) obtained "spore-like" bodies from several additional strains of milky-disease bacteria. They achieved this by stabilizing the vegetative cells through the use of special, nutritious media and reducing the oxygen supply and temperature when the cell population reached maximum. The refractile bodies were not heat-resistant, but it would appear that they may be at an early stage in spore development.

It appears that there is a very low percentage of strains of *B. popilliae* and *B. lentimorbus* and, apparently, the other milky-disease organisms as well that have the inherent genetic makeup to permit them to sporulate on artificial media, if conditions are favorable. Among such conditions are a sufficiently high cell population and a reduced oxygen tension, such as provided by Costilow, Sylvester, and Pepper (5) or by Steinkraus and Tashiro (25). The research of Haynes and Rhodes (9) and Rhodes, Roth, and Hrubant (16) shows how sensitive even a strain with the inherent ability to form spores on artificial media can be to changes in concentrations and sources of nutrients. Particularly, the effect of activated

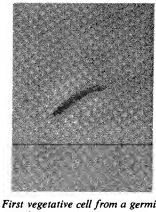

FIG. 8. *First vegetative cell from a germinated spore of Bacillus popilliae.*

carbon (9) would suggest that there may be inhibitors to sporulation present in most laboratory media.

No reports of successful repetition of the Steinkraus and Tashiro (25) two-stage cell paste method of obtaining sporulation have appeared in the literature. Also, our attempts to obtain sporulation by the procedures described by Rhodes et al. (16) and Costilow et al. (5) have been futile. However, we have not used the same strains of milky-disease organisms. Generally, we have been at most only two or three subtransfers away from the spores produced in vivo. The other workers have generally used vegetative cell inocula which have been carried on artificial media for a number of subtransfers (and lyophilized).

As soon as the spores are germinated on artificial media, those cells most saprophytic are selected in preference over those cells most parasitic in nature. Further subculture reinforces the saprophytic nature, and, even if a portion of these cells remain infective via injection, there is evidence that these cells, even if they sporulate, lose some of their ability to parasitize their normal host via ingestion (25).

It would appear that strains are all important in the study of the milky-disease organisms. This, in turn, suggests that genetic makeup may hold the key to answers to the many questions still unanswered. It must be recognized that the milky-disease strains vary widely in their ability to grow, both in vivo in different hosts and in vitro on various media. If the host is changed or the medium is modified, these changes are impressed upon or reflected by the organisms that emerge. The fact that all the milky-disease species and strains can parasitize at least one species of beetle larvae and that they show similar fermentation patterns on artificial media indicate that they are all closely related, even if they do differ morphologically.

So far, it has not been possible to duplicate the profuse in vivo sporulation on artificial media. Furthermore, in the only studies in which spores formed in vitro have been studied for virulence via the normal pathway of ingestion by healthy larvae (25), the virulence of the artificially produced spores was much lower, i.e., 16 versus 92% infectivity in spores produced in vivo. Thus, not only must a method be developed for the production of spores in quantity outside the host, but also the vital factor enabling the microorganism to penetrate from the gut into the hemocoel must be retained.

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